Exopolysaccharide production by *Bifidobacterium longum* BB-79

Cynthia M. Roberts, W.F. Fett[1], S.F. Osman[1], C. Wijey[1], J.V. O'Connor[1] and D.G. Hoover
Department of Food Science, University of Delaware, Newark, DE and [1]U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, PA, USA

4986/07/94: received 15 July 1994 and accepted 6 December 1994

C.M. ROBERTS, W.F. FETT, S.F. OSMAN, C. WIJEY, J.V. O'CONNOR AND D.G. HOOVER. 1995. *Bifidobacterium longum* BB-79 produced an acidic extracellular polysaccharide (EPS), especially when grown on solid medium. The EPS was isolated by ethanol precipitation followed by dialysis and lyophilization. Anion exchange and gel-filtration chromatography were used to further purify and characterize the EPS. The average molecular weight was greater than 200 kDa as estimated by chromatography. Based on gas-liquid chromatography (GLC) and GLC-mass spectrometry analyses, the EPS appears to be composed of galactose and an unidentified hexose (possibly glucose) with a carboxyethyl (lactic acid) substituent. Lactose, when used as the primary carbon source in liquid media, gave the highest yield of EPS. Incubation times longer than 24 h and the initial culture pH (pH 6·0–9·0) had little effect on the amount of EPS produced.

INTRODUCTION

The human large intestine contains a complex microflora. Bifidobacteria are a normal part of the gut microbiota of healthy humans and are often the predominant species, especially in breast-fed infants (Hoover 1993). The genus *Bifidobacterium* encompasses a group of anaerobic, Gram-positive bacteria that mainly produce lactic and acetic acids as fermentation end-products (Scardovi 1986). *Bifidobacterium longum*, along with the very closely related species *B. infantis*, are commonly isolated from the faeces of adults and infants. Bifidobacteria have been reported to have a wide range of therapeutic values in humans (Mitsuoka 1989). The health and nutritional benefits ascribed to bifidobacteria include maintenance of a healthy intestinal microflora, improvement of lactose digestibility and tolerance, antitumourigenic activity, reduction of serum cholesterol levels, synthesis of 'B-complex' vitamins and absorption of calcium (Hughes and Hoover 1991). In countries other than the US, pharmaceutical companies market preparations containing bifidobacteria for therapeutic purposes (Biavati *et al.* 1992).

Currently, there is significant interest in adding beneficial micro-organisms to various foodstuffs to create what are now called functional foods or nutraceuticals. Bifidobacteria are now added to a variety of fermented dairy products in Europe and Japan and their adaptation to foods is just beginning in the US. Outside the US cows' milk modified with 'bifidus factors' is sold for the purpose of promoting the growth of indigenous bifidobacteria in the human intestinal tract.

Correspondence to: Dr D.G. Hoover, Department of Food Science, University of Delaware, Newark, DE 19716-3301, USA.

Bacteria which produce exopolysaccharide (EPS) are used to some extent in fermented dairy products. Mucoid variants of lactococci are currently used in the preparation of Scandinavian fermented milks (Cerning 1990; Roller and Dea 1992). The dairy industry's interests in EPS are for their possible use to ensure increased viscosity, texture improvement and enhanced smoothness of mouthfeel (Cerning *et al.* 1986). Although EPS-producing bacterial cultures are now readily available for commercial use, the composition of EPS from dairy cultures and the cultural factors affecting EPS synthesis have rarely been studied.

The production of an EPS is a relatively novel characteristic in the genus *Bifidobacterium*. *Bifidobacterium longum* BB-79 was isolated by the Morinaga Milk Industry Co. (Japan) and found to produce slimy colonies, especially when grown on media containing 5% fermentable sugar. In this report we present information on the composition of the EPS produced by *B. longum* BB-79 as well as the effect of carbon source, time of incubation and initial culture pH on its synthesis.

MATERIALS AND METHODS

Culture conditions

Bifidobacterium longum BB-79 was obtained from N. Ishibashi (Morinaga Milk Industry Company, Ltd, Zama City, Japan). The culture was stored in glycerol at $-60°C$ until use. After thawing, a 1% (v/v) inoculum was placed into 10 ml sterile MRS broth (Difco Laboratories, Detroit, MI, USA). The broth culture was incubated at 37°C for 16–18 h in a Coy anaerobic chamber (Ann Arbor, MI, USA) that contained a mixture of 85% N_2, 10% CO_2, and 5% H_2. The culture was then diluted in sterile peptone water (0·1%, w/v) to give a bacterial concentration of approximately 10^5 cfu ml^{-1}. The diluted culture (0·1 ml $plate^{-1}$) was spread on culture dishes (100 × 15 mm) containing MRS agar amended with 3% (w/v) lactose. The cultures were incubated anaerobically at 37°C for 3 d.

EPS isolation and purification

Mucoid growth was harvested from the agar surface using a minimal amount of distilled water and a bent glass rod. The suspension was collected, stirred vigorously and centrifuged at 15 000 *g* for 25 min. The supernatant fluid was removed and the cells washed with distilled water and recentrifuged. The combined supernatant fluids were recentrifuged to remove any remaining cells.

The EPS was precipitated by adding three volumes of chilled 95% ethanol to the combined supernatant fluids. After standing overnight at 4°C, the resultant precipitate was collected by centrifugation (10 500 *g*, 20 min), the EPS pellets were dissolved in distilled water and KCl was added to a final concentration of 1% (w/v). This procedure was repeated three times. The final precipitate was collected by centrifugation. The EPS pellet was dissolved in and dialysed against distilled water at 4°C for 36 h and lyophilized. These preparations are referred to as crude EPS.

Analytical methods

All chemicals and reagents were obtained from Sigma unless otherwise noted. The protein content was estimated either by use of the Bio-Rad protein assay kit, following the microassay procedure provided by the manufacturer, or by determining the absorbance at 280 nm. Bovine serum albumin was used as standard. Total neutral carbohydrate content was determined by the method of Dubois *et al.* (1956), with D-glucose as standard. Uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen (1973), with D-mannuronic acid lactone as standard. Hexosamine content was determined by the method of Johnson (1971), with D-glucosamine as standard. The presence of ketose sugars was determined by the method of Dische (1962), with D-fructose as standard. The presence of acetate or succinate was determined as described by McComb and McCready (1957), with glucose pentaacetate as standard. An enzymic assay utilizing lactate dehydrogenase (Jeanes *et al.* 1976) was used to test for pyruvate.

Ion-exchange chromatography

Crude EPS was subjected to anion-exchange chromatography on a column (2·5 × 18 cm) of DEAE-Sepharose CL-6B(Pharmacia, Piscataway, NJ, USA). The column was equilibrated in 20 mmol l^{-1} sodium phosphate buffer, pH 6·8. Crude EPS (30 mg) was dissolved in buffer and loaded onto the column bed. The equilibration buffer (300 ml), the linear 0–2 mol l^{-1} NaCl gradient (500 ml) in equilibrium buffer and 2 mol l^{-1} NaCl (50 ml) in equilibrium buffer were used sequentially for elution at a flow rate of 0·5 ml min^{-1}. Fractions (4·6 ml) were collected and tested for neutral carbohydrate by colorimetric assay.

Gel-filtration chromatography

Crude EPS samples were subjected to gel-permeation chromatography on a column (2·5 × 42 cm, total bed volume of 206 ml) of Sephadex G-200 (Pharmacia). The void volume was determined using blue dextran. After equilibrating the column with three bed volumes of 100 mmol l^{-1} NaCl, 10 mg of crude EPS dissolved in 4 ml of 100 mmol l^{-1} NaCl was loaded, and elution was carried out with three bed volumes of 100 mmol l^{-1} NaCl at 0·16 ml min^{-1}. Fractions (4 ml) were collected and tested for neutral carbohydrate content by colorimetric assay. Peak fractions were combined, dialysed against distilled water and lyophilized. The lyophilized sample was weighed and the neutral sugar content determined by colorimetric assay.

Gas-liquid chromatography (GLC) and GLC-mass spectrometry (MS) analyses

Extracellular polysaccharide samples (2 mg ml^{-1}) were hydrolysed in 1 mol l^{-1} sulphuric acid for 1·5 h at 100°C. Hydrolysed samples were neutralized by addition of $BaCO_3$, clarified by centrifugation and taken to dryness under a stream of N_2 gas. Aldononitrile acetate derivatives were prepared by the procedure of Varma *et al.* (1973). Derivatized samples were analysed on a Hewlett-Packard model 5880A gas-liquid chromatograph (flame ionization detection) fitted with a SP-2330 (15 m, 0·25 mm i.d.) capillary column (Supelco, Bellefonte, PA, USA). The oven temperature was programmed from 150° to 250°C at 4°C min^{-1}.

GLC-MS analyses were done on a Hewlett-Packard 5989A mass spectrometer interfaced with a Hewlett-Packard 5890 gas chromatograph. GLC conditions were the same as previously described. Mass spectra were obtained in the electron impact mode and the chemical ionization mode, with ammonia as the reactant gas. The temperature of the ionization source and the GLC/MS interface was 250°C.

Effect of carbon source on EPS yield

Fresh overnight bacterial culture (2·5 ml) was placed into 250 ml sterile M17 broth (Difco) amended with 3% (w/v) sucrose, glucose, fructose or lactose. The cultures were incubated anaerobically at 37°C for 5 d. The resultant culture fluids were centrifuged at 2200 *g* for 20 min to loosely pellet the cells. The supernatant fluid was collected with a pipette and the cells washed once with distilled water. The EPS was precipitated from the combined supernatant fluids with 2 volumes of chilled 95% ethanol. After standing overnight at 4°C, the precipitate was collected by centrifugation (2200 *g*, 5 min), the pellet dissolved in distilled water, dialysed against distilled water at 4°C, lyophilized and weighed. The neutral sugar content of the samples was determined by colorimetric assay. As a control, the same procedure was carried out on fresh media without an added carbon source. The experiment was repeated twice.

Effect of incubation time and pH on EPS yields

A fresh overnight bacterial culture (10 ml) was placed into 1 l of sterile M17 broth amended with 3% lactose contained in a 2800-ml flask. The culture was incubated anaerobically at 37°C. On days 1, 2, 3, 5, 7 and 10, a 100-ml aliquot was aseptically removed. One ml of culture was serially diluted in sterile 0·1% (w/v) peptone water and MRS agar-spread plates were used to determine viable cell counts. The EPS was isolated from the remaining 99 ml of culture and examined following the same procedures used in the carbon source experiments. The experiment was done twice.

The initial pH experiments were conducted similarly as noted above. Media were adjusted to a pH of 6·0, 8·0 and 9·0 with concentrated HCl or KOH as required.

RESULTS

Characterization of EPS

Three separate preparations of crude EPS were analysed. The average yield of crude EPS per culture dish was approximately 0·67 mg. Colorimetric assays indicated the presence of neutral sugar (approximately 30–40% by weight), acetate or succinate (3% by weight) and the absence of both ketoses and hexosamines. The enzymic assay for pyruvate was negative, and levels of both uronic acid and protein were low (<5%). Analysis of hydrolysates of crude EPS samples by GLC and GLC-MS indicated the presence of glucose, galactose and an unknown component in the approximate ratio of 1 : 1·5 : 0·6 based on peak areas. Under the GLC conditions used, the unknown component eluted approximately 10 min after galactose.

Crude EPS was further purified by anion exchange chromatography. A minor amount of material containing neutral sugar eluted with the initial buffer wash (Fig. 1). A major peak (I) was obtained using a continuous NaCl gradient followed by a smaller peak (II) which eluted with the 2 mol l^{-1} NaCl wash. Fractions corresponding to peaks I and II, as well as neutral sugar-containing fractions eluting with buffer, were combined, dialysed against distilled water and lyophilized. The dry weight for the peak I sample was 9·8 mg and for the peak II sample 1·1 mg. No weight was obtained for the material eluting with buffer alone due to the extremely small amount of lyophilized material obtained. The neutral sugar contents of peaks I and II were 34% and 22%, respectively. Neither of the peak materials contained uronic acid detectable with the colorimetric assay. Analysis of the samples by GLC indicated that both samples contain glucose, galactose and the unknown compound in the relative ratios of 1 : 10 : 2 for peak I and 1 : 5 : 2 for peak II based on peak areas.

An estimation of molecular weight was obtained by gel-filtration chromatography of crude EPS. For each of the two column runs, only a single peak of neutral sugar-containing materials was obtained which eluted at the column void volume (80 ml; data not shown). As indicated by colorimetric assays, the peak samples contained approximately 36% neutral sugar and were devoid of protein. As determined by GLC and GLC-MS analyses, the composition of samples corresponding to the single peaks was similar to crude EPS with glucose, galactose and the unknown compound present.

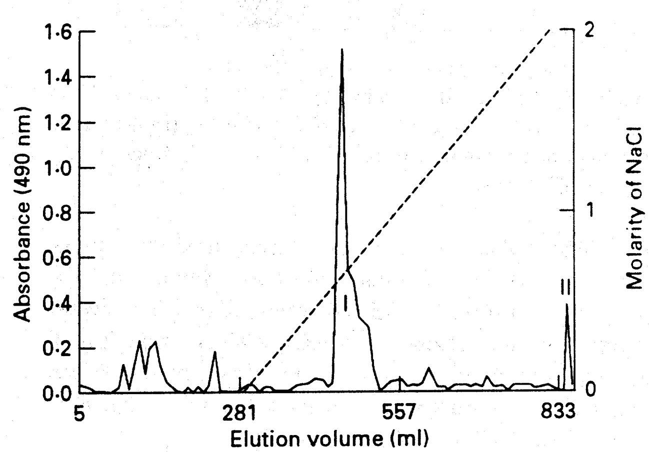

Fig. 1 Anion-exchange chromatography of crude extracellular polysaccharide from *Bifidobacterium longum* BB-79 on DEAE-Sepharose CL-6B. Fractions diluted with buffer alone or with a NaCl gradient (– – –) were assayed for total carbohydrate content (——)

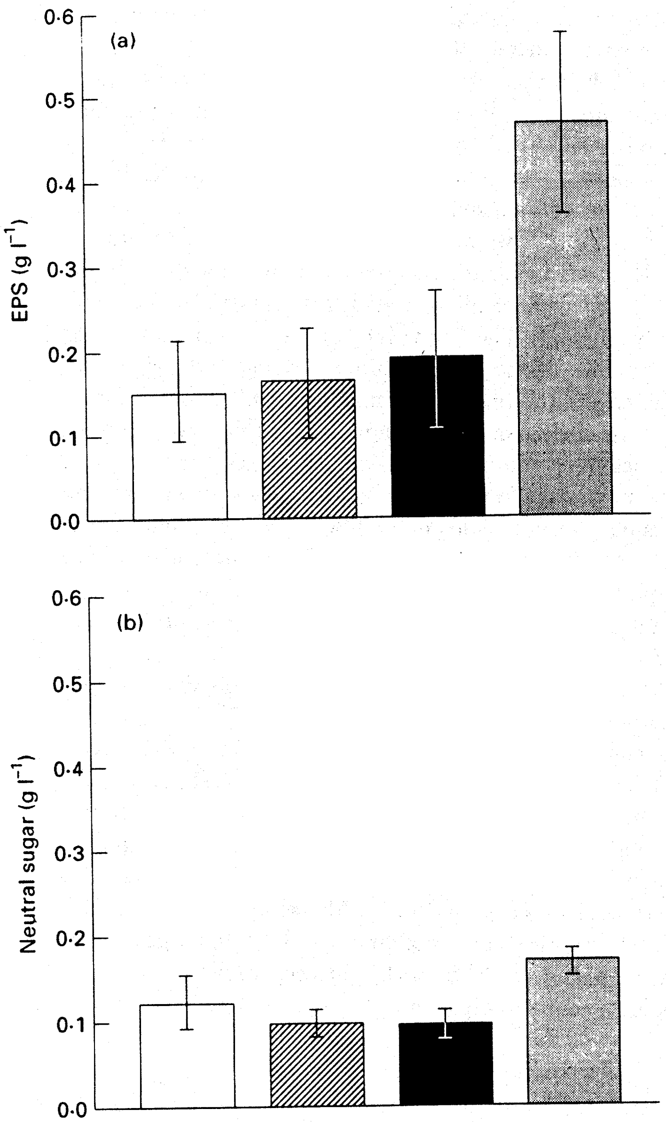

Fig. 2 Effect of carbon source (3%) on extracellular polysaccharide (EPS) production by *Bifidobacterium longum* BB-79 in M17 broth. (a) Dry weight of ethanol precipitates; (b) neutral sugar content of ethanol precipitates. □, Sucrose; ▨, fructose; ■, glucose; ▩, lactose

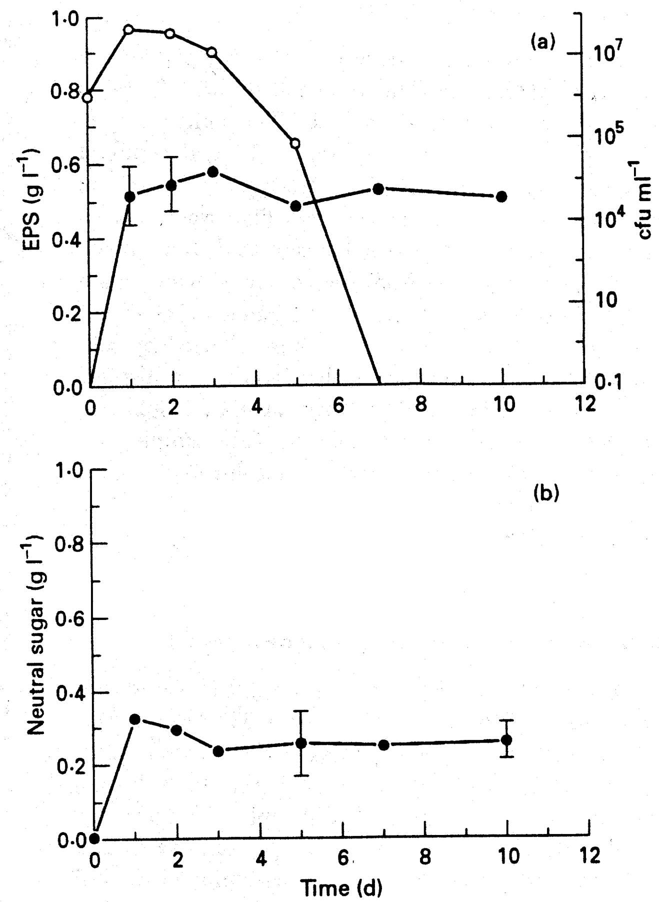

Fig. 3 Effect of incubation time on extracellular polysaccharide (EPS) production and cell growth by *Bifidobacterium longum* BB-79 in M17 broth with 3% lactose. (a) Dry weight of ethanol precipitates. ●, EPS (g l⁻¹); ○, cfu ml⁻¹. (b) Neutral sugar content of ethanol precipitates

GLC-MS analyses of the late eluting unknown compound indicated an apparent molecular weight of 357 ($[M]^{+\cdot} = 358$, observed). This corresponds to the molecular weight of the lactone of a carboxyethyl substituted hexose. Ions observed in the electron impact mass spectra are consistent with this structure (*m/z* 315[M − 42], *m/z* 255 [M − 102] and *m/z* 187).

Effect of carbon source, incubation time and initial culture pH on EPS yield

The effect of carbon source on EPS yield is shown in Fig. 2. The dry weights of ethanol-precipitable material obtained for the various media alone and the neutral sugar content of this material were subtracted from the values shown. Lactose gave the highest yield of crude EPS both in terms of weight (0·466 g l^{-1}) and in glucose equivalents (0·168 g l^{-1}). The amounts of EPS produced from sucrose, fructose and glucose as the primary carbon source in the media were similar. Since lactose gave the highest EPS yield, M17 broth amended with 3% lactose was chosen to conduct studies on incubation time and initial pH.

The time of incubation had little effect on resultant yields of EPS (Fig. 3). Optimal EPS yields were obtained after 24 h. Cell viability counts dropped slowly over the first 3 d of incubation, followed by a precipitous decline in cell numbers. No viable cells were detected on the 7th day and thereafter. The initial pH of the culture medium in the range 6·0–9·0 also had little effect on EPS yields (Fig. 4); however, the culture medium with an initial pH of 6·0 did

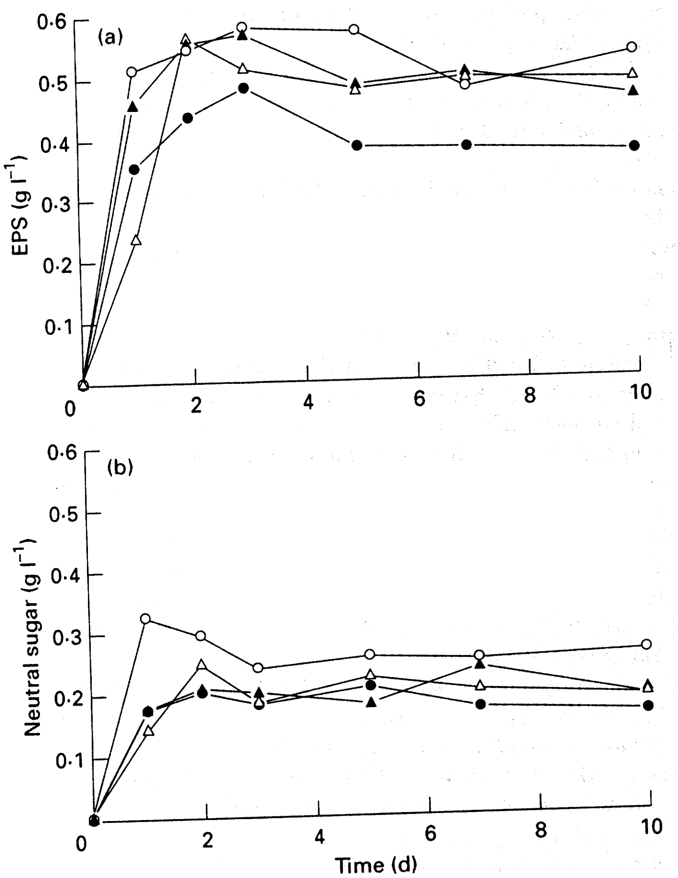

Fig. 4 Effect of initial culture medium pH on extracellular polysaccharide (EPS) production by *Bifidobacterium longum* BB-79 in M17 broth. (a) Dry weight of ethanol precipitates; (b) neutral sugar content of ethanol precipitates. ●, pH 6·0; ○, pH 6·9; ▲, pH 8·0; △, pH 9·0

show reduced production of EPS throughout the 10-d period. The pH of all four culture media dropped to between 3·9 and 4·2 after 10 d.

DISCUSSION

There are few reports on EPS production by strictly anaerobic bacteria (Sutherland 1990) and to our knowledge, this is the first report on the composition of an EPS from a bifidobacterium. *Bifidobacterium longum* BB-79 produced a high molecular weight, acidic, EPS in culture, whose synthesis was affected by the primary carbon source of the culture medium.

The EPS from *B. longum* BB-79 apparently had a molecular weight >200 kDa because this is the exclusion limit of Sephadex G-200 for dextrans (Pharmacia). A sodium chloride solution was used for elution in order to inhibit polymer aggregation. The primary components of the EPS based on GLC and GLC-MS analyses were galactose and an unidentified sugar which has been tentatively identified as a lactic acid derivative of a hexose. A carboxyethyl substituent has previously been described as a component of EPS from bacteria (Sutherland 1990; Osman and Fett 1993). The presence of a carboxyethyl substituent would explain the acidic nature of the polymer in the absence of uronic acids. The different ratios of galactose and the unknown compound seen for the two EPS samples obtained after anion-exchange chromatography may be due to variable formation of a lactone from the carboxyethyl-substituted hexose during sample derivatization (Osman and Fett 1993). The peak II material may be more highly substituted with lactic acid leading to a higher charge density. The low amounts of glucose observed in the purified preparations may have resulted from partial hydrolysis of the ether bond of the substituted sugar if glucose is the hexose. Unsubstituted glucose is most likely not part of the polymer, since this would require an unusually large repeating unit. Most EPS from bacteria have repeating units of two to five monomers (Sutherland 1990).

Several other lactic acid-producing bacteria have been reported to produce EPS (Cerning 1990). These EPS can be polymers composed of a single type of sugar (homopolysaccharides) or of several sugars (heteropolysaccharides). Examples of homopolysaccharides are the glucans named mutan and dextran produced by *Streptococcus mutans* and *Leuconostoc mesenteroides*, respectively. The sugar composition of heteropolysaccharides produced by lactic acid bacteria is a subject of some controversy, but normally glucose and galactose are identified, with galactose predominating (Cerning 1990).

Bifidobacterium longum BB-79 produced the highest amount of EPS when lactose was used as the primary carbon source in liquid media (0·466 g of crude polymer l^{-1}). This represented an approximate 200–300% increase in yield over EPS production in media containing sucrose, fructose or glucose. Optimum production of EPS in the presence of lactose may indicate that EPS would be produced by growth of BB-79 in fermented milk products.

Extracellular polysaccharide was produced during the active growth phase as indicated by the fact that there was no significant increase in EPS production after day 1. The relatively constant amount of EPS recovered from days 2 to 10 suggests that no EPS-depolymerizing enzymes were synthesized. Initial culture medium pH had little effect on EPS production. The slight decrease in EPS yields in pH 6·0 medium during incubation may have been due to reduced culture biomass.

There are several possible benefits that cultures of *B. longum* intended for use as a probiotic might derive from production of an EPS. A protective coating of EPS may allow the bacterium to better withstand stomach acid and bile salts. In the intestinal tract, EPS production may improve adherence to the intestinal mucosa and increase longevity in the intestinal tract. The acidic EPS could also play a role in the increased uptake of calcium ascribed to colonization with bifidobacteria, by acting as an ion-exchange medium (Oda *et al.* 1983; Doco *et al.* 1990). In addition, the future generation of stable EPS-overproducing mutants of bifidobacteria might lead to improvements in the texture and/or mouthfeel of fermented dairy products.

ACKNOWLEDGEMENTS

The authors thank Dr N. Ishibashi (Morinaga Milk Industry Co.) for strain BB-79, and the National Dairy Promotion and Research Board for partial support of this project. Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

REFERENCES

Biavati, B., Sgorbati, B. and Scardovi, V. (1992) The genus *Bifidobacterium*. In *The Prokaryotes* Vol. I. ed. Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H. pp. 816–833. Baltimore: Springer-Verlag.

Blumenkranz, N. and Absoe-Hansen, G. (1973) New method for quantitative determination of uronic acids. *Analytical Biochemistry* **54**, 484–489.

Cerning, J. (1990) Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiology Reviews* **87**, 113–130.

Cerning, J., Bouillanne, C., Desmazeaud, M.J. and Landon, M. (1986) Isolation and characterization of exocellular polysaccharide produced by *Lactobacillus bulgaricus*. *Biotechnology Letters* **8**, 625–628.

Dische, Z. (1962) Color reaction of ketoses with carbazole and sulfuric acid. In *Methods in Carbohydrate Chemistry* Vol. I. ed. Whistler, R.L. and Wolfrom, M.L. pp. 481–482. New York: Academic Press.

Doco, T., Wieruszeski, J.M., Fournet, B., Carcano, D., Ramos, P. and Loones, A. (1990) Structure of an exocellular polysaccharide produced by *Streptococcus thermophilus*. *Carbohydrate Research* **198**, 313–322.

Dubois, M., Giles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 349–356.

Hoover, D.G. (1993) Bifidobacteria: Activity and potential benefits. *Food Technology* **47**, 120–124.

Hughes, D.B. and Hoover, D.G. (1991) Bifidobacteria: Their potential use in American dairy products. *Food Technology* **45**, 74–83.

Jeanes, A., Rogovin, P., Cadmus, M.C., Silman, R.W. and Knutson, C.A. (1976) Polysaccharide (xanthan) of *Xanthomonas campestris* NRRL B-1459: Procedures for culture maintenance and polysaccharide production, purification, and analysis. Bulletin ARS-NC-51. US Department of Agriculture.

Johnson, A.R. (1971) Improved method of hexosamine determination. *Analytical Biochemistry* **44**, 629–635.

McComb, E.A. and McCready, R. M. (1957) Determination of acetyl in pectin and in acetylated carbohydrate polymers. Hydroxamic acid reaction. *Analytical Chemistry* **29**, 819–821.

Mitsuoka, T. (1989) *Microbes in the Intestine*. Tokyo, Japan: Yakult Honsha Co., Ltd.

Oda, M., Hasegawa, H., Komatsu, S., Kambe, M. and Tsuchiya, F. (1983) Antitumor polysaccharide from *Lactobacillus* sp. *Agricultural Biological Chemistry* **47**, 1623–1625.

Osman, S.F. and Fett, W.F. (1993) Structure of the acidic exopolysaccharide of *Pseudomonas marginalis* ATCC 10844. *Carbohydrate Research* **242**, 271–275.

Roller, S. and Dea, I.C.M. (1992) Biotechnology in the production and modification of biopolymers for foods. *Critical Reviews of Biotechnology* **12**, 261–277.

Scardovi, V. (1986) Genus *Bifidobacterium*. In *Bergey's Manual of Systematic Bacteriology* Vol. 2. ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1418–1434. Baltimore: Williams & Wilkins.

Sutherland, I.W. (1990) *Biotechnology of Microbial Exopolysaccharides*. New York: Cambridge University Press.

Varma, R., Varma, R.S. and Wardi, A.H. (1973) Separation of aldononitrile acetates of neutral sugars by gas-liquid chromatography and its application to polysaccharides. *Journal of Chromatography* **77**, 222–227.